ISSN: 2394-1391

Indian Journal of Biology
Volume 4 Number 1, January - June 2017
DOI: http://dx.doi.org/10.21088/ijb.2394.1391.4117.5

Original Article

Optimization of Carbon Sources for the Amylase Production and Growth of *Bacillus licheniformis* JAR-26 under Submerged Fermentation

Nand Lal*, Jeevan Jyoti*,, Priti Sachan***

Abstract

Amylases (EC 3.2.1.1, 1, 4-α-D-glucan glucanohydrolase) are one of the most important and oldest industrial enzymes that hydrolyze starch at α-1, 4 glycosidic bond in the interior of the starch molecule, and hold the maximum market share of enzyme sales. Amylases are ubiquitously produced by plants, animals and microorganisms, however, microbial sources are the most preferred for large scale production and industrial use. The production of α- amylases from microbes depends on the strain, physical (pH, temperature, aeration) and nutritional (carbon, nitrogen, mineral ions) factors. Keeping this in view, the present study aimed to investigate effect of different concentrations (control, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0% w/V) of five carbon sources *viz* starch, glucose, maltose, fructose and sucrose on growth and enzyme production from *B. licheniformis* JAR-26. Among the tested carbon sources, maltose proved best carbon source for amylase production and maximum amylase production was recorded at 2% maltose (4.181 U/ml of medium). At this concentration growth/OD was 1.529 and bacteria could utilize 98.2% of the available sugar in the medium. After maltose, starch was second suitable source for enzyme production and 2% starch showed 3.622 U/ml enzyme production. Glucose and fructose resulted in higher biomass yield (maximum biomass at 2% glucose, OD=1.741) in comparison to other sources but amylase production was very poor (lowest at 4% fructose, 0.710 U/ml). From comparison of the various treatments, it is suggested that for maximum biomass production of *B. licheniformis* JAR-26, growth medium may be supplemented with 3% glucose whereas to achieve maximum amylase production culture medium may be supplemented with 2% maltose under submerged fermentation.

Keywords: Amylase Production; Submerged Fermentation; *Bacillus licheniformis*; Carbon Source; Maltose.

Introduction

Amylases are widely used starch hydrolyzing enzymes with the maximum market share (about 30%) of the total enzymes sales. α-Amylases (EC 3.2.1.1; α-4-glucan glucano-hydrolase) are calcium containing endoamylases catalyze hydrolysis of starch and related carbohydrates by randomly cleaving internal α-D (1-4) glycosidic linkage, yielding glucose, maltose, maltotriose, and other oligosaccharides (Ryan, 2011). Although amylases can be obtained from higher plants and animals also, the enzymes from microbial sources, particularly grown in extreme environments, prove useful for industrial processes/demand due to its widespread use in the food, brewing, textile, detergent and pharmaceutical industries. Moreover, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry in addition to widespread use in starch saccharification and distilling industries.

Author's Affiliation: *Department of Life Sciences, C.S.J.M. University, Kanpur – 208024, India. ***Present*: Asmara College of Health Sciences, Asmara, Eritreaa, NE, Africa.

Reprint's Request: Nand Lal, Department of Life Sciences, C.S.J.M. University, Kanpur – 208024, Uttar Pradesh India.
E-mail: nl_pr@yahoo.co.in

Received on 02.02.2017, **Accepted on** 23.02.2017

The major advantage of using microorganisms for the production of amylases is continuous economical bulk production and their relatively easy genetic

©Red Flower Publication Pvt.Ltd

manipulation to obtain enzymes of desired characteristics. A significant increase in amylase production and utilization occurred in early 1960s with the advent of *Bacillus subtilis* α-amylase and *Aspergillus niger* glucoamylase for the production of dextrose from starch as alternative to acid hydrolysis. Today, a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. Several *Bacillus* species (*Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus stearothermophilus*) are among the versatile producer of α-amylases (Naidu and Sharanraj, 2013). The two bacterial strains *Bacillus amyloliquefaciens* and *Bacillus licheniformis* have been exploited on the industrial scale (Alariya *et al.*, 2013). Production of amylases is influenced by physical (pH, temperature, aeration etc.) and nutritional factors (carbon, nitrogen, mineral ions etc.) which control the growth and metabolism of producer organism (Halder *et al.*, 2014; Abel-Nabey and Farag, 2016). Nutritional factors in particular play vital role for the commercial production of bacterial amylases (Dutta *et al.*, 2016). The nature and concentration of carbon and nitrogen source in the culture medium are reported to important factors governing bacterial growth and amylase production (Akeel and Umar, 2010; Lal *et al.*, 2016).

Soils receiving the biodegradable kitchen wastes are one of the rich sources of starch degrading microorganisms as they are rich in starchy substances. *B. licheniformis* JAR-26 is an acidophilic and thermostable extracellular α-amylase producing acidophilic bacteria isolated from soils rich in spoiled tomatoes as kitchen waste (Jyoti *et al.*, 2011). Keeping in view the role of nutritional factors on bacterial growth and amylase production, the present study aims to investigate the effects of various concentrations (0, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0% w/V) of different carbon sources namely Starch, Glucose, Maltose, Fructose and Sucrose on amylase production, growth and sugar utilization by *Bacillus licheniformis* JAR-26.

Material and Methods

Microorganism

Starch hydrolyzing *Bacillus licheniformis* JAR-26 was isolated from spoiled tomatoes and collected in sterilized stoppered glass vials.

Media and Chemicals

Starch, Yeast extract, Peptone, $MgSO_4.7H_2O$, KH_2PO_4, NaCl, $CaCl_2$, Agar, Distilled H_2O, Phosphate buffer, Iodine solution, 3, 5 dinitrosalicylic acid (DNS), Glucose, Maltose, Fructose, Sucrose, Sephadex G-100, DEAE-Cellulose (DE-52), CM-Cellulose, Acrylamide, Bis-acrylamide, N,N,N′n′-tetramethylethane-1,2-diamine (TEMED), Sodium dodecyl sulphate (SDS), Ammonium persulphate.

Isolation of Microorganism

The thermostable, acidophilic starch hydrolyzing bacteria (*B. licheniformis* JAR-26) was screened for extracellular acidophilic amylase production by using starch medium containing (g/L): Starch (Merck, Germany), 10.0; yeast extract, 5.0; peptone, 2.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5; NaCl, 1.5; $CaCl_2$, 0.1; Agar, 20.0. Initial pH was adjusted to 5.5. One gram of each sample was suspended in 9.0 ml of sterile water and 0.1 ml of suitably diluted suspension was spread on the agar plates. The plates were incubated at 45, 50, 55 and 60 °C for 24 to 48 h. The isolated colonies were flooded with iodine solution and colonies bearing good colorless halos around them were picked and maintained on starch agar slants at 4 °C and further assessed for enzyme production in liquid medium. The characterization and identification of the isolate was made following Bergey's Manual of Systemic Bacteriology. The method of identification used was as given by Collee *et al.* (1996).

Amylase Production

The basal fermentation medium for enzyme production contained (g/L): Starch, 10.0; yeast extract, 5.0; peptone, 5.0; KH_2PO_4, 0.12; $CaC1_2.2H_20$, 0.12; $MgSO_4.7H_20$, 0.12; $MnSO_4.4H_20$, 0.02. Initial pH of the medium was adjusted at 5.5 and 50 ml of medium in 250 ml of Erlenmeyer flasks were inoculated with a cell suspension of optical density 0.5 (prepared from 24 h old culture). All the flasks were incubated for four days on a rotary shaker (Remi) at 170 rpm at 45 °C. Samples were drawn after a time interval of 12 h, centrifuged at 8000 Xg for 10 minutes and cell free culture supernatant fluid was used as enzyme source.

Assay of Enzyme

Culture filtrate (Supernatant) was used for assessing enzymatic activity by the method of Srivastava and Baruah (1986). One ml of 1% (w/V) starch (Merck, Germany) solution was taken in test tube and 0.2 ml of 0.2 M phosphate buffer (pH 5.5) and 0.2 ml of deionized water was added to it. The

mixture was equilibrated at 70 °C for 10 minutes in a water bath. 0.1 ml of supernatant was added and then reaction was stopped by adding 1.0 ml of 3, 5 dinitrosalicylic acid (DNS). The mixture was heated and the color intensity was measured at 540 nm (Bernfield, 1955) using a spectrophotometer (Systronics Spectrophotometer 169). One unit of amylase activity was defined as the amount of amylase that liberates 1.0 mg of glucose per minute under assay conditions. In all the above experiments the enzyme activity was calculated as the average of three independent sets of experiments (the standard deviation in all cases was found negligible).

Effect of Carbon Source

The basal fermentation medium was supplemented with the different concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0% w/V) of carbon sources i.e. starch, glucose, maltose, fructose and sucrose and its effect was recorded on bacterial growth, amylase production and sugar utilization by test bacteria.

Results and Discussion

The test organism was isolated from spoiled tomatoes and screened by zone hydrolysis method (Figure 1) and later identified as *Bacillus licheniformis* JAR-26 (Figure 2) according to Bergey's Manual of Determinative Bacteriology (Jyoti *et al.,* 2011).

Fig. 1: Starch Hydrolysis by isolate JAR-26

Fig. 2: *Bacillus licheniformis* JAR-26

Carbon forms backbone of all biomolecules (protein, lipid, carbohydrates, nucleic acids etc.) and is required relatively in large amounts than other nutrients in the production medium. Starch is most widely accepted carbon nutrient source for induction of microbial amylolytic enzymes, hence, 1% (w/V) starch was considered as reference and essential component of basal fermentation medium. Effects of different carbon sources (starch, maltose, glucose, sucrose and fructose) and their concentrations on α-amylase production, growth and sugar utilization by *Bacillus licheniformis* JAR-26 are summarized in Figure 3, 4 and 5, respectively, and all the parameters

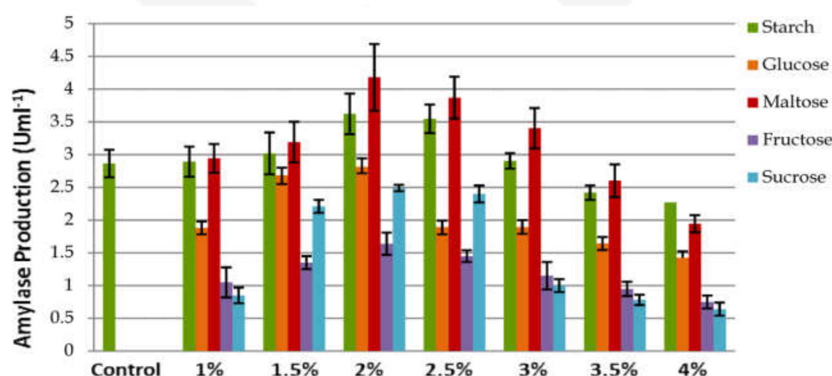

Fig. 3. Effect of different concentrations of carbon source(s) on Amylase Production by *B. licheniformis*

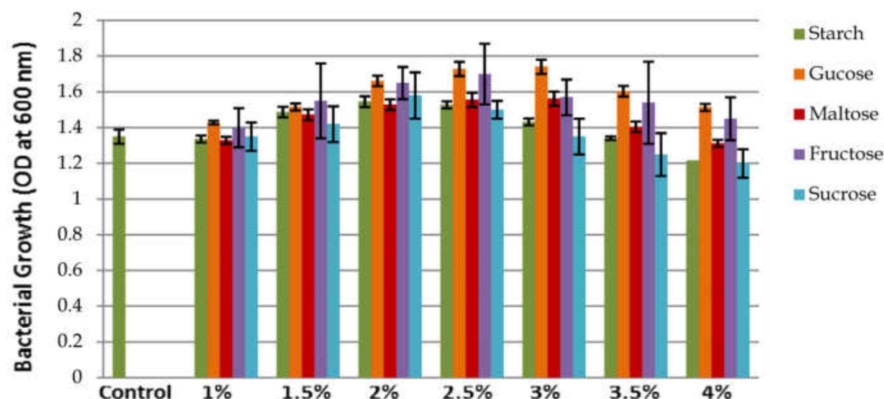

Fig. 4. Effect of different concentrations of carbon source(s) on growth of *B. licheniformis*

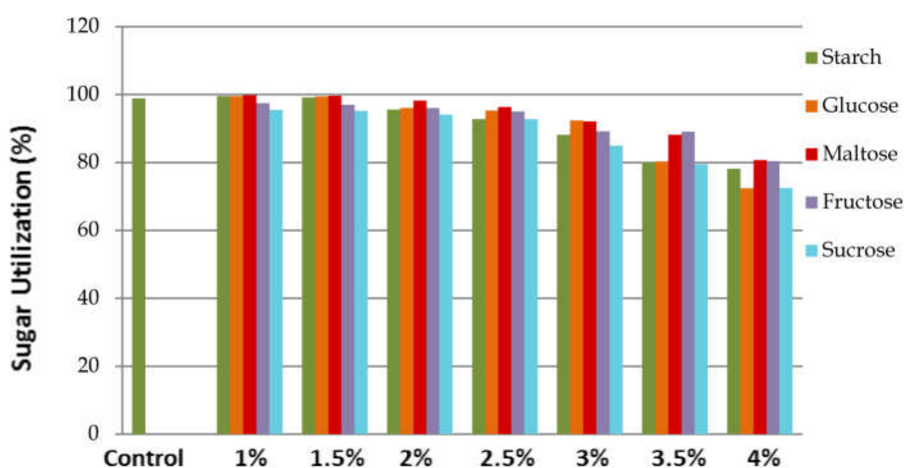

Fig. 5. Effect of different concentrations of carbon source(s) on sugar utilization by *B. licheniformis*

varied with the nature and concentration of carbon source used in the medium.

As shown in Figure 3, none of the tested carbon source enhanced the enzyme production by *B. licheniformis* except maltose and starch over control medium. Even amylolytic activity was inversely affected by glucose, fructose and sucrose with the amylase production and was recorded lower than control. Maltose was found to be the most suitable carbon source for amylase production and maximum amylase production was achieved at 2.0% Maltose (4.181 Uml^{-1}). At 2.0% Maltose, growth was 1.529 in terms of OD at 600 nm and bacteria could utilize 98.2% of available sugar in the medium. Similar findings are reported by Thippeswamy *et al.* (2006) in *Bacillus* species (B_3) where highest amylase production (0.464 Uml^{-1}) was induced by maltose. Suribabu *et al.* (2014) also found maltose superior than other carbon sources tested for amylase production with *Brevibacillus borostelensis* R1 under submerged fermentation.

Starch proved to be second best carbon source for amylase production by *Bacillus licheniformis* JAR-26. Starch at 2% showed 3.622 Uml^{-1} amylase productions and growth was 1.546 in terms of OD at 600 nm and the bacteria could utilize 99.6% of available sugar in the medium. Starch is a generally accepted nutritional component for induction of amylolytic enzymes. Bajpai and Bajpai (1989) observed enhanced production of α-amylase when *Bacillus megaterium* grown on starch containing medium. Mishra and Behera (2008) also reported 2% starch concentration as suitable carbon source for amylase production as well as bacterial growth in *Bacillus* species.

It can be inferred from the Figure 4 that Glucose and Fructose resulted in higher biomass yields, 1.75 at 3.0% Glucose and 1.70 at 2.5% Fructose, respectively, which was superior over the values observed in the present study for Maltose and Starch. On the contrary, amylase production was too poor (2.814 Uml^{-1} & 1.64 Uml^{-1}, respectively) with glucose and fructose. Haseltine *et al.* (1996) during studies on hyperthermophilic archaebacteria *Sulpholobus*

solfataricus reported that glucose represses amylase production and inhibits expression of amylase gene and same seems to be true for *B. licheniformis* JAR-26. Sucrose turned to be least preferred carbon source for *B. licheniformis* JAR-26 for all the three parameters i.e. amylase production, growth and sugar utilization except 2.5% where amylase production exceeded over corresponding glucose and fructose concentrations. Utilization of sucrose by bacteria is routed through its conversion into glucose and fructose and among these two monosaccharides glucose is the most preferred substrate. Equal concentrations of glucose and sucrose did not show corresponding performance as the glucose produced from sucrose by hydrolysis remains in lesser amounts. Fructose turned closer to glucose and exhibited nearly similar effects/trends on growth and sugar utilization but for amylase production sucrose turned superior over fructose in the range of 1.5 to 2.5 % (w/V). This observation on fructose and sucrose for amylase production is in conformity with the observations of Suribabu *et al.* (2014) on *Brevibacillus borostelensis* R1. In a study on *B. flavothermus* by Kelly *et al.* (1997), the presence of sucrose, fructose and glucose in the media resulted in good bacterial growth with little or no amylase production. On contrary to findings of present study, Sreekanth *et al.* (2013) reported 2% glucose to increase amylase activity significantly (66 Uml^{-1}) among various tested carbon sources.

Sugar utilization by *B. licheniformis* in the presence of various tested carbon sources is shown in figure 5 and varied for bacterial ability to utilize carbon in different forms. It has been reported that the synthesis of carbohydrate degrading enzymes including amylase (s), in most of the microbial species in general and the genus *Bacillus* in particular, is subjected to catabolic repression by readily metabolisable substrates such as glucose and fructose (McMahon *et al.*, 1999; Suman and Ramesh, 2010) like most other inducible enzymes and the same turns to be true in present study.

Conclusion

The production of amylases by microbes is known to be affected by a variety of physiochemical factors i.e. composition of the growth medium, inoculum age, pH, temperature, carbon and nitrogen source and mineral elements. From the present study it can be concluded that the bacterial isolate *B. licheniformis* JAR-26 produces significant amount of amylases (4.181 Uml^{-1}) at 2% maltose concentration in culture medium. Starch proved to be second most suitable carbon source with 3.662 Uml^{-1} amylolytic activities at 2% concentration. The role of glucose, fructose and sucrose was found less significant than other carbon sources (maltose and starch).

References

1. Abel-Nabey HM, Farag AM. Production, optimization and characterization of extracellular amylase from halophilic *Bacillus licheniformis* AH214. African J Biotechnol 2016; 15:670-683.
2. Akeel BM, Umar DM. Effect of Alternative Carbon and Nitrogen Sources on Production of Alpha-amylase by *Bacillus megaterium*. World Appl Sci J 2010; 8:85-90.
3. Alariya SS, Sonia S, Gupta S, Gupta BL. Amylase activity of a starch degrading bacteria isolated from soil. Arch Appl Sci Res 2013; 5:15-24.
4. Bajpai P, Bajpai PK. High-temperature alkaline á-amylase from *Bacillus licheniformis* TCRDC-B13. Biotechnol Bioengg 1989; 33:72-78.
5. Bernfeild P. Amylases á and â. In Methods in Enzymology, Colowich SP, Kaplon NO, editors, 1955; 1:149-158.
6. Collee JG, Fraser AG, Marmion BP, Simmons A (eds). Mackie and McCartney Practical Medical Microbiology, 14th edition. Churchill Livingstone, New York, 1996.p.317-327.
7. Dutta P, Deb A, Majumdar S. Optimization of the medium for the production of extracellular amylase by the *Pseudomonas stutzeri* ISL B5 isolated from municipal solid waste. Int J Microbiol 2016 (http://dx.doi.org/10.1155/2016/4950743).
8. Halder D, Biswas E, Basu M. Amylase production by *Bacillus cereus* strain BRSC-S-A26MB under optimal laboratory condition. Int J Curr Microbiol Appl Sci 2014; 3:1035-1047.
9. Haseltine C, Rolfsmeier M, Blum P. The glucose effect and regulation of alpha amylase synthesis in the hyperthermophilic archaeon *Sulpholobus solfataricus*. J Bacteriol 1996; 178:945-950.
10. Jyoti J, Lal N, Lal R, Kaushik A. Partial Purification and Characterization of an Acidophilic Extracellular á-Amylase from *Bacillus licheniformis* JAR-26. Int J Adv Biotechnol Res 2011; 3:315-320.
11. Kelly CT, Bolton DJ, Forgaty WM. Biphasic production of á-amylase of *Bacillus flavothermus* in batch fermentation. Biotechnol Lett 1997; 19: 675-677.
12. Lal N, Jyoti J, Sachan P. Optimization of Nitrogen Source(s) for the Growth and Amylase Production from *Bacillus licheniformis* JAR-26 under Submerged Fermentation. Indian J Biol 2016; 3:127-132.
13. Mishra S, Behera N. Amylase activity of a starch

degrading bacteria isolated from soil receiving kitchen wastes. African J Biotechnol 2008; 7: 3326-3331.

14. Naidu MA, Sharanraj P. Bacterial Amylase: A Review. Int J Pharm Biol Sci Arch 2013; 4:274-287.
15. Ryan SM, Fitzgerald GF, Sinderen D. Screening for identification of starch, amylopectin and pullulan degrading activities in *Bifidobacteria* strains. Appl Environ Microbiol 2011; 72:5289-5296.
16. Sreekanth MS, Vijayendra SVN, Joshi GJ, Shamala TR. Effect of carbon and nitrogen sources on simultaneous production of α-amylase and green food packaging polymer by *Bacillus* sp. CFR 67. J Food Sci Technol 2013; 50:404-408.
17. Srivastava RAK, Baruah JN. Culture conditions for production of thermostable amylase by *Bacillus stearothermophilus*. Indian J Microbiol 1981; 21: 131-139.
18. Suman S, Ramesh K. Production of a thermostable extracellular amylase from thermophilic *Bacillus* species. J Pharm Sci & Res 2010; 2:149-154.
19. Suribabu K, Govardhan TL, Hemalatha KPJ. Influence of carbon sources on α-amylase production by *Brevibacillus* sp. under submerged fermentation. Int J Res Engg Technol 2014; 3:292–299.
20. Thippeswamy S, Girigowda K, Mulimani VH. Isolation and identification of á-amylase producing *Bacillus* sp. from dhal industry waste. Indian J Biochem Biophys 2006; 43:295-298.